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249^{ser} TP53 mutation in plasma DNA, hepatitis B viral infection, and risk of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) from regions with high dietary exposure to aflatoxins and endemic for hepatitis B virus (HBV) often contain a specific mutation at codon 249 in TP53 (249^{ser}; AGG to AGT, Arg to Ser). This mutation is also detectable in circulating cell-free DNA from the plasma of HCC patients and healthy subjects in these regions. We have examined the joint effect of plasma 249^{ser} and HBV infection in a case–control study design involving 348 control, 98 cirrhotic, and 186 HCC participants from The Gambia, West Africa, an area of high HCC incidence. The 249^{ser} mutation was detected in 3.5% of controls, 15.3% of cirrhotics, and 39.8% of HCC cases (adjusted odds ratios (OR): 4.83, (95% confidence interval (CI): 1.71–13.7) for cirrhosis and 20.3 (8.19–50.0) for HCC). HBsAg positivity along with plasma 249^{ser} was observed in 45/183 (24.6%) HCC cases compared to only one (0.3%) control. Risk for HCC was associated with markers of HBV alone (OR: 10.0, 95% CI: 5.16–19.6), 249^{ser} alone (OR: 13.2, 95% CI: 4.99–35.0), and both markers present (OR: 399, 95% CI: 48.6–3270). These results suggest a multiplicative effect on HCC risk resulting from the mutational effect of aflatoxin on TP53, as monitored by detection of plasma 249^{ser}, with concomitant chronic infection with HBV.
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Introduction

Hepatocellular carcinoma (HCC) incidence rates vary greatly worldwide, generally reflecting the prevalence of HCC etiologic factors, namely hepatitis B (HBV) and hepatitis C (HCV) virus infection and exposure to high levels of aflatoxin in the diet (IARC, 1994, 2002). The highest HCC incidence rates are observed in areas of eastern Asia and of sub-Saharan Africa, regions with a high chronic HBV prevalence and where approximately 60–65% of HCC are attributed to HBV (Pisani *et al*., 1997). The prevalence of HCV also varies worldwide, but the estimated fraction of HCC attributable to HCV (around 20–25%) is similar between less and more developed regions (Pisani *et al*., 1997). It is also well documented that exposure to AFB1 is causally associated with HCC (IARC, 2002); however, the attributable HCC risk to this carcinogen and the degree of interaction with viral hepatitis remains unclear. In The Gambia, West Africa, HCC is the most common cancer in men and second in women (Bah *et al*., 2001), with a median age of 45 years at presentation. HBV transmission occurs horizontally among young children and 15–20% of adults become HBV chronic carriers (Vall Mayans *et al*., 1990; Whittle *et al*., 1990). The prevalence of HCV infection is low (Mendy *et al*., 1998) while aflatoxin exposure is ubiquitous (Hudson *et al*., 1992; Wild and Hudson *et al*., 1992).

Aflatoxins are mycotoxins produced by species of *Aspergillus* that frequently contaminate staple foods. Aflatoxin B1 (AFB1), one of the most potent hepatocarcinogens known, is the predominant form of exposure in humans (Wogan, 2000). In rodents exposed to AFB1 at doses similar to those occurring in humans, levels of aflatoxin–albumin adduct in the serum have been correlated to levels of liver-specific DNA damage and to development of HCC (Wild *et al*., 1996). In humans, the implication of aflatoxin as a hepatocarcinogen is based on ecological correlations of HCC rates with the aflatoxin content of foods or with the detection of adducts in blood or urine (reviewed in Groopman *et al*., 1996; Jackson and Groopman, 1999). In a sentinel nested case–control study within a large male Chinese cohort, Qian *et al*. (1994) reported increased risk of HCC with HBV infection alone and with urinary aflatoxin adducts alone, while the combined effect of both factors suggested a multiplicative effect. However, most studies of aflatoxin/HBV interactions were limited in evaluating joint effects since the populations studied

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consisted almost exclusively of HBV carriers (Chen *et al*., 1996; Wang *et al*., 1996; Sun *et al*., 1999). Previous studies in Africa are limited to ecological studies comparing regional HCC rates to estimated aflatoxin intake (Autrup *et al*., 1987; Omer *et al*., 1998; van Rensburg *et al*., 1990). Three small case–control studies of HCC in Africa have assessed aflatoxin exposure at the individual level, with equivocal results (Olubuyide *et al*., 1993; Mandishona *et al*., 1998; Omer *et al*., 2001). In the Gambia, levels of aflatoxin–albumin adducts in the serum have been validated to reflect the intake of foods contaminated with AFB1 (Hudson *et al*., 1992; Wild and Hudson *et al*., 1992). Extensive population-based surveys have demonstrated the presence of serum aflatoxin–albumin adducts in over 95% of Gambians (Wild *et al*., 1990; Wild and Hudson *et al*., 1992). Exposure to AFB1 starts in the perinatal period, through *in utero* transfer (Wild and Shrestha *et al*., 1992) and breast-feeding (Wild *et al*., 1987) and continues throughout life from consumption of peanuts (or groundnuts, as termed locally). Temporal patterns of aflatoxin–albumin adduct levels correlate with the seasonal availability of groundnuts (Wild *et al*., 2000).

HCC pathogenesis involves the accumulation of genetic alterations (reviewed in Thorgeirsson and Grisham, 2002; Wang *et al*., 2002), such as in *p16/CDKN2A* (deletions, hypermethylation; in up to 50% of the cases), *β-catenin* (mostly mutations in exon 3, 20–30%) *cyclins A* and *D* (overexpression, 20–30%), and *M6P/IGF2R* (mutations, 18–33%). Alterations of the *TGF-β* signalling pathway genes (*Smad 3, Smad 4*) have been described in up to 10% of HCC. Mutations in *TP53* show striking differences in prevalence and pattern depending on geographic area (Hainaut and Hollstein, 2000). In high incidence areas, *TP53* is mutated in over 50% of HCC and over half of these are a single missense, hotspot mutation at codon 249 (AGG to AGT,) resulting in the substitution of a serine for an arginine (249ser). This mutation is extremely uncommon in HCC from USA and Western Europe and in cancers other than HCC (Montesano *et al*., 1997). The sequence context of codon 249 (AGGCC) represents a site of intermediate affinity for the formation of AFB1-induced lesions. After AFB1 metabolization by several CYP450 enzymes (mainly 1A2 and 3A4) in hepatocytes, a primary DNA adduct is formed (8–9, dihydro-8-[*N*7-guanyl]-9-hydroxyaflatoxin), which is naturally converted to two secondary lesions, an apurinic (AP) site and a stable, AFB1-formamidopyrimidine (AFB1–FAPY) adduct, the latter considered as the most mutagenic (Smela *et al*., 2002). Slow repair at codon 249, perhaps due to the interference of HBxAg with the host's repair system or to specific pro-oncogenic effects of 249ser on liver cells, may contribute to the biological selection of 249ser as a single 'hotspot' mutation in the pathogenesis of HCC (Ponchel *et al*., 1994; Smela *et al*., 2001; Friedler *et al*., 2003).

Low levels of cell-free DNA can be isolated from the plasma of healthy individuals; these levels are often increased in patients with cancer or autoimmune diseases (Wu *et al*., 2002; Anker *et al*., 2003). The process by which DNA occurs in the plasma is unclear but may involve enhanced lysis or apoptosis of tumor cells, shedding some of their contents in the blood stream. In a preliminary study, we have shown that that 249ser could be detected in plasma DNA from a proportion of HCC cases in The Gambia but not in HCC cases from Europe (Kirk *et al*., 2000). We have also found that there was a concordance of 88% between the presence of 249ser in the biopsy and in the plasma of the same HCC patient (Szymanska *et al*., 2004). In the present study, we have determined the presence of 249ser in the plasma of over 600 subjects enrolled in a case–control study in The Gambia, to evaluate the individual and joint effects of plasma 249ser and HBV markers on HCC development.

Results

Characteristics of study groups

Demographic and virologic information on participants is presented in Table 1. The mean age of HCC cases at presentation was 48.5 years while cirrhotic patients were on average 5.2 years younger. Similar to other areas with high HCC incidence rates, male HCC cases outnumbered females by more than four to one. Cirrhotic patients were younger and more likely to be female than HCC cases. The mean age and gender distribution of controls fell between HCC cases and

Table 1 Characteristics of study participants[a]

	Controls		*Cirrhotics*		*HCC cases*	
	No.	*%*	*No.*	*%*	*No.*	*%*
Age[b]	44.7 (15.3)		43.2 (14.6)		48.5 (15.1)	
Gender						
Males	243	69.8	64	65.3	151	81.2
Females	105	30.2	34	34.7	35	18.8
Ethnicity						
Mandinka	112	32.6	23	24.0	49	26.9
Fula	76	22.1	28	29.2	42	23.1
Wollof	53	15.4	22	22.9	39	21.4
Other	103	29.9	23	24.0	52	28.6
HBV status						
HBsAg neg	296	85.8	44	44.9	74	40.4
HBsAg pos	47	13.6	40	40.8	88	48.1
HBeAg pos	2	0.6	14	14.3	21	11.5
Anti-HCV status						
Neg	318	97	84	94.4	131	78.9
Pos	10	3.0	5	5.6	35	21.1
Groundnut intake[c]						
1 or less/week	67	23.5	20	25.3	45	28.7
2 to 6/week	79	27.7	27	34.2	34	21.7
Once daily	87	30.5	18	22.8	44	28.0
More than daily	52	18.3	14	17.7	34	21.7

[a]Study groups include 348 control, 98 cirrhotic and 186 HCC participants. Deviations from these totals are due to missing data for some participants. [b]Mean age (s.d.), in years. [c]In number of servings

cirrhotics (Table 1). HBV infection was evident in 101 of 175 (57.7%) HCC cases; 88 (48.1%) were HBsAg-positive only and 21 (11.5%) were HBsAg- and HBeAg-positive. Cirrhotic patients displayed an intermediate prevalence of HBV and HCV seromarkers compared to HCC cases and controls. Among the cirrhotic and control groups, 54 of 98 (55.1%) and 49 of 345 (14.2%) participants, respectively, had at least one marker of chronic HBV present. Antibodies to HCV (anti-HCV) were detected in 35 of 166 (21.1%) HCC cases, five of 89 (5.6%) cirrhotics, but in only 10 of 328 (3.0%) control participants.

Detection of 249ser TP53 mutations in plasma

Figure 1 demonstrates the detection of 249ser by RFLP. Digestion of wild-type DNA generates two bands of 92 and 66 bp, whereas mutant material, in which the restriction site has been destroyed, yields a band of 158 bp (Figure 1). A second *Hae*III restriction site present in the PCR product but located outside the coding area of exon 7 provided a positive control for digestion. The final assessment of mutation status was performed by bidirectional sequencing of a PCR product generated by reamplification of the mutant-specific (uncut) band with nested primers (see Methods).

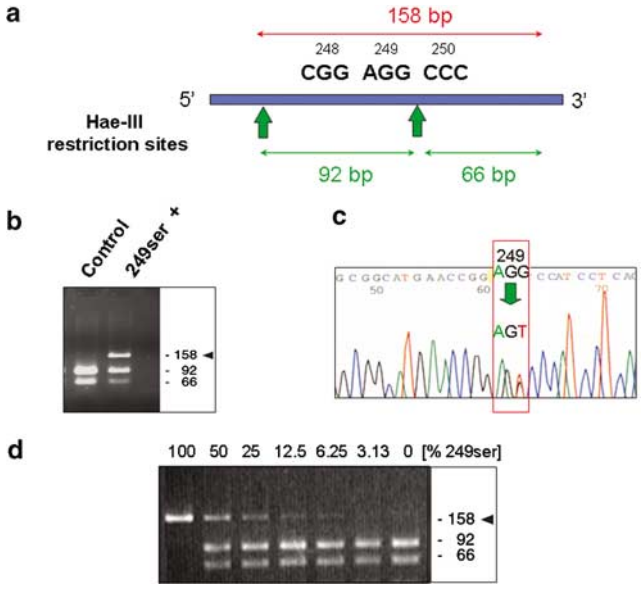

Figure 1 Example of detection of plasma 249ser TP53 mutation in HCC cases and controls from The Gambia. (**a**) TP53 exon 7 was amplified by PCR and mutation at codon 249 was identified by restriction digestion. Exon 7 contains two *Hae*III sites, including one (GGCC) spanning codons 249 and 250. A mutation at the second or third base of codon 249 or at the first or second base of codon 250 destroys this restriction site. (**b**) Presence of an undigested, 158 bp fragment (arrowhead) is indicative of mutation. Lane 1: negative control with wild-type bands at 66 and 92 bp; lane 2: HCC case with 158 bp band indicative of 249ser TP53 mutation. (**c**) Automated DNA sequencing of TP53 exon 7 corresponding to the HCC case analysed in (**b**). The sequence of codon 249 is boxed. Only a portion of the electropherogram is shown. (**d**) Sensitivity of mutation detection by restriction digestion. DNA isolated from the liver cancer cell line PLC/PRF5 that contains two 249ser TP53 mutant alleles was diluted in DNA from normal human lymphocytes in proportions as indicated, amplified by PCR, and subjected to restriction analysis. The lower limit for detection of a signal corresponding to mutant DNA was observed at a ratio of 6.25% of mutant DNA in 93.75% of wild-type DNA

Extensive studies were performed to determine the specificity and sensitivity of 249ser mutation detection by RFLP. Negative (wild-type genomic DNA) and positive (DNA of PLC/PRF/5 cells, ATCC, No. CRL-8024, homozygous for 249ser mutation) controls were included in all restriction analyses. The sensitivity of the method was evaluated using dilutions of PLC/PRF/5 DNA. The restriction digestion could detect mutant material at a minimum rate of about 3–6% as compared to wild-type sequence (Figure 1). Using the same dilutions of cell-line DNA, the method was benchmarked against other procedures available for detection of 249ser in plasma DNA. RFLP was found to be marginally less sensitive than short oligonucleotide mass analysis (SOMA); (Jackson *et al*., 2001), which detected down to 1% of mutant material (M Friesen, manuscript in preparation), but RFLP was substantially more sensitive than standard mutation detection methods such as temporal temperature gradient electrophoresis (TTGE) or denaturing high pressure liquid chromatography (DHPLC), which did not detect less than 15% mutant material (E Caboux, data not shown).

Since the amount of circulating DNA is known to vary with certain disease states, we quantified the amount of total DNA in plasma. Figure 2 presents the DNA concentrations (data transformed onto a $\log_{10}$ scale) by study group and by 249ser status. HCC cases did have higher concentrations of total plasma DNA (mean 0.17 ng/ml) compared to controls (0.049 ng/ml, $P=0.02$) but were statistically similar to cirrhotic participants (0.10 ng/ml, $P=0.61$). When stratified by 249ser status and study group (Figure 2), the mean plasma DNA concentration was lower among 249ser positive controls ($P=0.02$) and tended to be higher among 249ser positive HCC cases ($P>0.05$) compared to their respective negative participants. No differences were observed by 249ser status among cirrhotic participants ($P=0.84$). Thus, in total, detection of 249ser was not uniformly related to plasma DNA concentration and was unlikely to account for observed differences in the prevalence of plasma 249ser detection between study groups.

Other exon 7 TP53 mutations

Through sequencing of exon 7, we observed 14 TP53 mutations other than 249ser in plasma DNA, five (35.7%) among HCC cases and nine (64.3%) among controls. Of these 14 mutations, six were at codon 249 (three AGA, two AGC, one AAG), six at codon 250 (three CTC, two TCC), and two at other sites (248 CTT, 254 GTC). There were four participants with two mutations each, involving a 249ser mutation and additionally a 249 AGA (two controls), a 250 TCC (HCC case), or a 254 GTC mutation (control). Among controls, nine of 21 (42.9%) TP53 mutations detected in plasma were non-249ser compared to only five of 79 (6.3%) non-249ser among HCC cases ($P<0.001$).

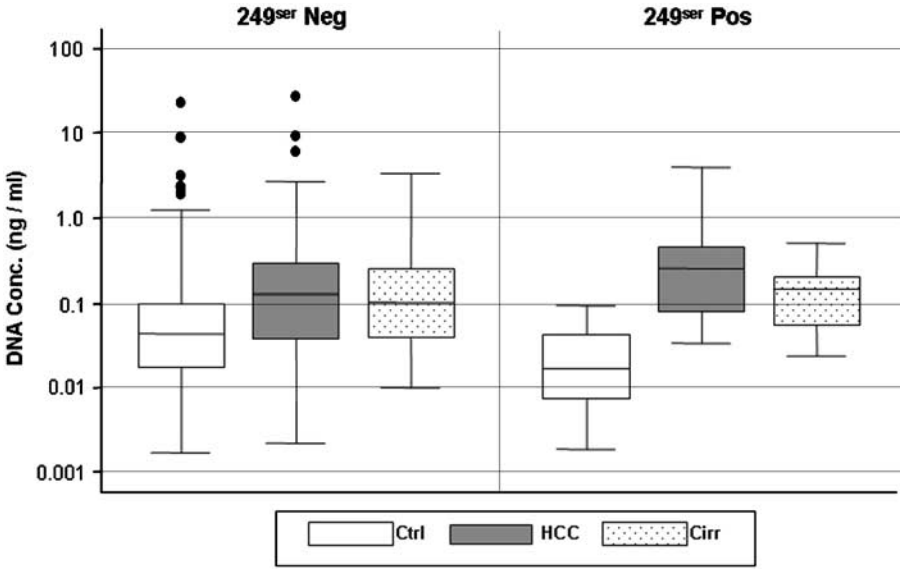

Figure 2 Box and whisker plots of cell-free DNA levels by study group and 249ser status. Total DNA concentration (in ng/ml, presented on $\log_{10}$ scale) was determined by pico-green flourescense methods. The line within the box denotes the median and the box spans the interquartile range (25–75th percentiles). Whiskers extend from the 10 to 90th percentiles with outliers represented by circles

Table 2 Determinants of 249ser TP53 mutations in plasma[a]

	249ser neg		*249ser pos*		*Adjusted OR*	
	No.	*%*	*No.*	*%*	*OR*	*95% CI*
Study group						
Control participants	336	96.6	12	3.5	1	—
Cirrhotic patients	83	84.7	15	15.3	3.34	(1.30–8.54)
HCC cases	112	60.2	74	39.8	20.6	(10.2–41.6)
Season of recruitment						
Nov–Jan	142	26.7	20	19.8	1	—
Feb–Apr	144	27.1	23	22.8	0.94	(0.42–2.08)
May–Jul	107	20.2	34	33.7	3.07	(1.44–6.59)
Aug–Oct	138	26.0	24	23.8	2.00	(0.89–4.50)
Groundnut intake						
Less than once daily	235	53.9	37	43.5	1	—
Once or more daily	201	46.1	48	56.5	1.79	(1.04–3.08)
AST level[b]						
<80 U/l	422	85.1	46	51.1	1	—
80 or >U/l	74	14.9	44	48.9	1.80	(0.96–3.38)
HBeAg status[c]						
Neg	129	84.9	40	74.1	1	—
Pos	23	15.1	14	25.9	2.67	(0.98–7.23)

[a]Multivariable model includes adjustment for study group, season of recruitment and daily groundnut intake. [b]The addition of aspartate aminotransferase (AST) levels or HBeAg status to this model was associated with improved predictive ability of borderline statistical significance. Additional variables evaluated but for which no association with plasma 249ser TP53 was found included: age, gender, recruitment site, place of residence, ethnicity, socio-economic status variables and HBsAg or anti-HCV status (data not shown). [c]Only HBsAg-positive individuals were tested for HBeAg

Determinants of 249ser TP53 mutations in plasma

A total of 632 participants were evaluated for plasma 249ser. Of those, 531 were found to be negative (at the 3% detection cutoff) and 101 were positive. Our initial univariate analysis focused on identifying factors associated with detection of 249ser. Plasma 249ser was most strongly associated with study group; detected in cell-free DNA in 12 of 348 (3.5%) control participants, 15 of 98 (15.3%) cirrhotic patients, and 74 of 186 (39.8%) HCC cases (Table 2). In subsequent multivariable analysis, there was a significant increase in risk

for 249ser detection associated with both cirrhosis and HCC diagnoses. HBeAg-positivity, a surrogate marker for HBV viral replication, was associated with a 2.7-fold increased adjusted risk for plasma 249ser while elevated AST levels, a biochemical marker for liver damage, had a 1.8-fold increased adjusted risk (Table 2). These findings were of borderline statistical significance with the lower CI for each just overlapping unity.

Daily groundnut intake and the season of recruitment were also significantly associated with plasma 249ser (Table 2). Among all study participants, those who ate groundnuts at least daily were almost twice as likely to be positive for 249ser (OR: 1.79 (95% CI: 1.04–3.08)). In a case–control study design, the effect of the disease can limit the usefulness of estimating dietary aflatoxin exposure. Therefore, dietary assessment of groundnuts focused on recent (prior 2 months) consumption with additional questioning to determine if lifetime patterns had changed due to illness. As expected, 161 of 202 (79.7%) HCC cases reported eating fewer groundnuts recently compared to their lifetime pattern of intake, while only 44 of 387 (11.4%) control participants responded similarly ($P<0.001$). Cirrhotic patients reported changes in dietary patterns similar to HCC cases. Despite the clear impact of disease on diet, only 30 of 614 (4.9%) participants reported never eating groundnuts in the preceding 2 months. Additionally, 47.7% of all study participants reported eating groundnuts at least daily during the preceding 2 months, and this proportion did not differ by study group (Table 1). In multivariable analysis, there was no association between self-reported groundnut intake and risk for either cirrhosis or HCC (data not shown).

The rates of detection and the levels of aflatoxin–albumin adducts in The Gambia show clear seasonal variations, which generally follow the availability of groundnuts as part of the diet (Wild *et al*., 2000). Levels of aflatoxin exposure are typically highest from November through March when groundnuts are plentiful following the harvest, and lower from May through September when groundnuts are scarce. We observed similar seasonal variation in groundnut intake, with a higher proportion of participants eating groundnuts at least daily following the fall harvest, peaking in February to April (55%) with the lowest proportion with daily intake in May through July (40%). Seasonal variation was also observed with plasma 249ser detection, although slightly out of phase with reported groundnut consumption. A lower prevalence of plasma 249ser was detected in November through March (20%) with a higher prevalence in May through July (34%). These patterns were most notable among HCC and cirrhotic patients, with the higher overall mutation prevalence. No significant temporal variation of other markers of HCC risk or potential confounders was observed (data not shown).

249ser TP53 mutations and risk for cirrhosis and HCC

In addition to identifying correlates of 249ser detection, we sought to identify factors associated with cirrhosis or HCC diagnoses and incorporated the plasma 249ser marker into separate models with cirrhosis or HCC as the outcome variable. Compared to control participants and adjusting for potential confounders, the 249ser TP53 mutation conferred an almost five-fold increased risk for cirrhosis (OR: 4.83, 95% CI: 1.71–13.7) and a 20-fold increased risk for HCC (OR: 20.3, 95% CI: 8.19–50.0). In this same model, the OR's for HBV surface antigen and anti-HCV detection were (OR: 10.9, 95% CI: 5.6–21.3) and (OR: 3.87, 95% CI: 1.02–14.7) for cirrhosis and (OR: 20.2, 95% CI: 8.19–50.0) and (OR: 18.6, 95% CI: 6.53–52.9) for HCC, respectively.

Joint effect of HBV and 249ser TP53 mutations

Only one of 345 (0.3%) control participants was positive for both HBsAg and 249ser, compared to 45 of 183 (24.6%) HCC cases ($P<0.001$, Table 3). In a multivariable model incorporating combined exposure as a single categorical variable, HBsAg carriage (OR: 10.0, 95% CI: 5.16–19.6) and plasma 249ser (OR: 13.2, 95% CI: 4.99–35.0) were highly significant risk factors for HCC. When both HBsAg and plasma 249ser were detected, the OR was spectacularly increased to almost 400 (OR: 399, 95% CI: 48.6–3270). The joint effect of chronic HBV infection and the presence of the aflatoxin-associated 249ser in plasma was consistent with a greater than multiplicative effect; since the observed combined effect (399) is larger than the product of the two individual effects ($10 \times 13 = 130$). Among cirrhotic patients, 10 of 98 (10.2%) were both HBsAg and 249ser positive compared to <1% of controls; a similar pattern of markedly increased risk for cirrhosis (beyond the risk

Table 3 Joint effect of HBV infection and 249ser TP53 plasma mutation and association with HCC

	Controls		*HCC cases*		*Adjusted OR for HCC*[a]	
	No.	*%*	*No.*	*%*	*OR*	*95% CI*
HBV and TP53 status						
HBsAg (−)/TP53 (−)	287	83.2	56	30.6	1	—
HBsAg (+)/TP53 (−)	46	13.3	54	29.5	10.0	(5.16–19.6)
HBsAg (−)/TP53 (+)	11	3.2	28	15.3	13.2	(4.99–35.0)
HBsAg (+)/TP53 (+)	1	0.3	45	24.6	399	(48.6–3270)

[a]Multivariable model includes adjustment for age, gender, recruitment site and date, ethnicity, socio-economic status, HCV status and with HBV/TP53 status combined into a single categorical variable

Table 4 Comparison of demographic, laboratory and tumor characteristics in HCC cases by HBV and plasma 249ser TP53 mutation status[a]

	HBsAg (−)/TP53(−)		*HBsAg (+)/TP53(−)*		*HbsAg (−)/TP53(+)*		*HbsAg (+)/TP53(+)*	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Age[b]	54.9 (15.0)		41.3 (12.9)		61.4 (11.0)		41.6 (11.7)	
Male to female ratio	4.6 : 1		6.7 : 1		2.5 : 1		4.6 : 1	
Median AFP level	404		473		363		1000	
AST >80 (IU)	21	40.4	28	54.9	13	50.0	22	56.4
Anti-HCV positive	18	35.2	2	4.0	9	39.1	5	12.8
Multifocal HCC	36	66.7	29	54.7	18	64.3	31	70.5
Tumor volume >70%	21	38.2	22	42.3	10	35.7	16	36.4

[a]Represents all HCC cases from exposure strata presented in Table 3. There was no missing data for continuous variables; deviationsfrom these totals for categorical variables are due to missing data for some participants. [b]Mean age (s.d.), in years. [c]in ng/ml

with either independently) was observed among subjects with joint exposure (data not shown). No HCV-positive controls were also 249ser positive, so similar analysis of the joint HCV and 249ser effect was not possible.

Case-case comparisons by HBV and 249ser TP53 status

In stratified analysis comparing HCC cases by their HBV and plasma 249ser status, we observed several differences in demographic variables, laboratory markers, and tumor characteristics (Table 4). HCC cases with both HBV and 249ser tended to have higher median AFP levels and a higher proportion of multifocal disease at diagnosis compared to the other exposure strata. Other potential markers of advanced disease did not show differences (AST levels, estimated tumor volume); however, it should be noted that stage differences might have been difficult to identify because most cases presented with very advanced disease. The age of onset was notably younger among the HBV alone and HBV+/249ser+ HCC cases compared to HCC without either marker or with 249ser only. Similar to younger age of onset, the gender ratios displayed a greater male predominance among HBV-related HCC, although these differences were not statistically significant. These demographic comparisons were likely influenced by the underlying viral etiology, as HCV infection was significantly over-represented among both HBV negative groups ($P<0.01$).

Discussion

There is compelling evidence that the 249ser somatic change is a primary genetic event in hepatocarcinogenesis occurring in the context of high exposure to aflatoxin (reviewed in Montesano *et al*., 1997). First, there is substantial experimental data that show that the 249ser TP53 mutation is directly associated with DNA damage induced in hepatocytes by AFB1 (see Introduction). Second, of a total of 297 249ser mutations reported in the literature and compiled in the IARC TP53 mutation database (http://www.iarc.fr/p53, version R08), 196 (66%) have been detected in HCC. All but seven originate from regions where aflatoxin is a significant contaminant of the diet. In high-incidence areas of China (Jiangsu Province, Qidong County), 71 of the 80 (89%) TP53 mutations identified in HCC are 249ser**.** In sub-Saharan Africa, the prevalence of 249ser mutations in HCC has been addressed in four studies. In Mozambique (Ozturk, 1991), nine of 16 (56%) and in Senegal (Coursaget *et al*., 1993), 10 of 15 (67%) HCC cases had 249ser mutations detected. In The Gambia, we found 249ser in 10/29 HCC biopsies (35%) (Szymanska *et al*., 2004). These three countries are characterized by high exposure to AFB1. In contrast, a lower prevalence of 249ser was found in South Africa (6/32 HCC cases, 19%), a region of intermediate exposure to AFB1 (Bressac *et al*., 1991).

Research in The Gambia has documented HBV and HCV as independent causes of HCC, with attributable risk estimates of around 57% and 20%, respectively (Kirk *et al*., 2004). However, the contribution of exposure to aflatoxin is less well understood, due in part to the lack of valid and reliable markers for assessing relevant aflatoxin-induced DNA-damage. In this study, we show that plasma 249ser mutations were highly associated with HCC, with an estimated OR for HCC of 20, equivalent or greater than the risk reported with either HBV or HCV markers. There was a more moderate five-fold increased risk for ultrasound-defined cirrhosis associated with plasma 249ser. Our results suggest that detection of 249ser in the plasma is a potential marker of DNA-damage by aflatoxin, as it was strongly associated with cirrhosis and HCC and weakly associated with surrogate markers of both aflatoxin exposure (groundnut consumption) and of liver damage (AST levels).

The nature of combined exposure to HBV infection and aflatoxin continues to be an important research question. A meta-analysis of published 249ser TP53 mutations occurring in HCC tumor tissue confirmed that 249ser TP53 mutation prevalence was strongly correlated with regional aflatoxin exposure but not with the HBV status of the individual cases (Stern *et al*., 2001). In our study (Table 3), 106 of 171 (62%) HCC cases were HBsAg positive, 71 (42%) were 249ser TP53 mutation positive, and 44 (26%) were positive for both. One control participant was positive for both, allowing us to estimate the HCC risk, albeit with poor precision.

Study participants who were positive for HBsAg alone had an increased HCC risk of 10 (5.16–19.6). Those with the aflatoxin-associated 249ser TP53 mutation alone had an increased risk of 13 (4.99–35.0). Those who were HBsAg-positive and had the 249ser TP53 mutation had an estimated risk for HCC of around 400 (48.6–3270). These findings suggest a greater than multiplicative model of interaction between markers of chronic HBV carriage and aflatoxin on risk for HCC.

Experimental studies have suggested a biological interaction between HBV infection and aflatoxin. In woodchucks and tree shrews, animal species with a hepadnaviral liver pathology similar to that observed in HBV-infected humans, the administration of AFB1 resulted in a higher incidence of liver tumors than in the untreated but infected animals (Yan, 1989; Bannasch *et al*., 1995). More recently, similar findings have been reported in a transgenic mouse model positive for HBsAg and with or without a TP53 mutation at codon 246 (**AGGC**→**AGTC**), a sequence similar to that present in humans at codon 249. The presence of the TP53 mutation accelerated the tumorigenicity of AFB1 alone and greatly enhanced the cocarcinogenic effect observed in AFB1 treated HBsAg transgenic mice (Ghebranious and Sell, 1998). The exact, biological basis of this interaction is still poorly understood. Viral infection may increase DNA replication in the liver and/or change the pool size of the target cell population relevant to HCC development, thus maximizing the risk of converting a promutagenic lesion into a mutation. On the other hand, as discussed above, several viral components may interfere with the host's repair system and decrease DNA repair capacity.

Accumulating evidence suggests that prolonged viral replication is the primary factor in progression from HBV infection to chronic liver disease, fibrosis and cirrhosis, and in some individuals, HCC. Previously, we and others have documented marked increases in HCC risk associated with HBeAg, even beyond the risk seen with HBsAg (Yang *et al*., 2002; Kirk *et al*., 2004). While the worldwide prevalence of 249ser TP53 mutations in HCC does not differ by HBsAg status (Stern *et al*., 2001), prevalence differences by HBeAg status are unknown. In our study, we did not observe any difference in 249ser TP53 mutation prevalence by HBsAg status but did find a trend of increased 249ser TP53 among HBeAg cases. In adjusted analysis, HBeAg was associated with around a three-fold increased likelihood of 249ser detection. As we found no control participants positive for both HBeAg and the 249ser TP53 mutation, we could not estimate this joint effect.

Detection of HBeAg in serum is an indicator of active HBV replication and is associated with increased infectiousness, chronic 'active' hepatitis, and increased risk of HCC (Liang and Ghany, 2002). The effect of HBV infection on aflatoxin–albumin adducts levels in The Gambia was previously investigated. Among Gambian children 3–4 years of age, a higher geometric mean of aflatoxin–albumin adducts was seen in chronic HBV infected compared to uninfected children, with the highest levels in children with documented acute infection (Turner *et al*., 2000). In adults, however, statistically significant differences in adduct levels were not observed by HBV status (Wild *et al*., 2000). In The Gambia, there is rapid decay in HBV replication following childhood infection with rare persistence of HBeAg or HBV DNA detection into adulthood, except in individuals with severe liver disease or HCC (Mendy *et al*., 1999). The association between 249ser TP53 mutation and markers of HBV infection and replication may result from several possible mechanisms: (1) 249ser TP53 mutation may be more likely with the increased cell proliferation occurring in HBeAg positive carriers with chronic 'active' hepatitis, and mutated cells may provide a survival advantage; (2) aflatoxin may mediate, via immunosuppression or other pathways, increased HBV viral replication or HBeAg persistence. Further investigation is needed to fully define the mechanism of HBV and 249ser TP53 interaction.

The relationship of the plasma 249ser TP53 mutations that we detected to aflatoxin exposure is uncertain. As discussed previously, considerable evidence links the occurrence of the 249ser TP53 mutation directly to exposure to the carcinogen AFB1. We did not, however, try to correlate detection of 249ser TP53 mutations with detection of urinary aflatoxin adducts (an indicator of the preceding 1–2 days aflatoxin intake) or serum–aflatoxin–albumin adducts (an indicator of the preceding 2–3 months aflatoxin intake) in this case–control study. Detection of the 249ser TP53 mutation was only weakly correlated with a higher consumption of groundnuts, perhaps because the HCC cases had altered their diets or because the mutation reflects not the exposure level but rather the early biological effect of aflatoxin. Alternatively, as suggested by the seasonal variation of 249ser (Table 2), there might be a lag of some months between exposure to aflatoxin-contaminated groundnuts and detectability of 249ser TP53 mutation in cell-free plasma DNA. Large, longitudinal studies of healthy populations will be needed to clarify the relationships between diet, urine, or serum aflatoxin adducts and detection and quantification of 249ser TP53 mutation in plasma DNA.

We also observed additional exon 7 mutations within close proximity to codon 249, but this preferentially occurred among the control participants. Therefore, the specific accumulation of 249ser in HCC may reflect additional effects such as slower repair of promutagenic lesions at this position (perhaps due to the interference of viral infections with the host's repair system) or to specific pro-oncogenic effects of 249ser mutants in liver cells, resulting in their selection during the pathogenesis of HCC (Hussain and Harris, 2000; Smela *et al*., 2002).

Considerable evidence has shown that in various tumor types, the presence of mutations in plasma DNA reflects the presence of mutations present in these patients' original tumours (Anker *et al*., 2003; Taback and Hoon, 2004). With the mechanistic understanding of the role of specific DNA–AFB1 adducts and the resulting formation of 249ser mutations in hepatocytes (Smela *et al*., 2001), in conjunction with a significant majority of 249ser TP53 mutations reported in the

literature having been found in HCC (Hainaut and Hollstein, 2000), it is reasonable to deduce that the liver is the source of the 249ser TP53 mutation that we detected. When we examined a limited number (17) of matched liver tumor/plasma pairs, we found concordance between tumor and plasma DNA findings in 88% (15) of cases (Szymanska *et al*., 2004). Similarly high concordance has been reported by Jackson *et al*. (2001) among HCC cases from Qidong, China, a region with high HBV prevalence and AFB1 exposure. These findings strongly suggest that, in HCC patients, the presence of 249ser mutations in the plasma correlates with the clonal expansion of hepatocytes containing this mutation in the liver. It is evident that futher studies are required, in particular to determine whether the plasma 249ser mutation in healthy individuals and in patients with liver cirrhosis also originates from liver cells.

Whatever the source, we found that detection of the 249ser TP53 mutation in plasma DNA was highly associated with HCC, beyond the risk for HCC from HBsAg alone. Moreover, the presence of 249ser TP53 mutations in normal nontumorous liver tissues of HCC cases (Aguilar *et al*., 1994) and, as we report, in the plasma DNA of a few healthy individuals and significantly more cirrhotic patients supports the hypothesis that the mutation occurs early in the development of HCC. Recently, Groopman and co-workers have suggested that the 249ser TP53 mutation is detectable in the plasma of some HCC cases 1 to 5 years prior to HCC diagnosis (Jackson *et al*., 2003). Upon further evaluation, none of 12 control participants identified in our study as 249ser TP53 mutation positive had clinical, biochemical, or ultrasonographic evidence for occult HCC. In contrast to other types of cancer (Liu and Gelmann, 2002), we found limited evidence for stage differences in HCC by 249ser TP53 mutation status (Table 4); instead, most differences in tumor characteristics appeared to be most closely related to the underlying viral etiology. Prospective evaluation of nondiseased individuals is needed to determine whether detection of 249ser TP53 mutation has utility in identifying persons at high risk for developing cirrhosis or HCC.

As in other cancers, the accumulation of genetic and epigenetic events in a given cell population is the driving force leading to development of HCC. Payne *et al*. (1996) presented a dynamic cellular model that takes into account the long delay between primary HBV infection and the appearance of HCC many decades later. The liver comprises several populations of hepatic cells with varying potential to differentiate and/or to respond to exogenous insults. Quiescent stem cells, or 'oval cells' (Thorgeirsson, 1995), are postulated as precursor cells that, when modified by external insults, like HBV or aflatoxin, give rise to HCC (Sell, 1993; Strain and Crosby, 2000).

Our findings suggest a multiplicative effect on HCC risk resulting from chronic, active HBV replication and the mutational effect of AFB1 on codon 249 of the TP53 gene. In an environment like The Gambia, where AFB1 exposure begins shortly after or even before birth and where HBV infection occurs in early childhood, it is possible that the 249ser TP53 mutation occurs specifically in the mitotic liver stem cells of young children. In addition, other genetic alterations resulting from DNA oxidative damage produced during the chronic inflammatory process in HBV infection or chromosomal instability due to HBV integration into cellular DNA may also be occurring at this stage, and continuing in the case of chronic 'active' HBV carriers (see Hagen *et al*., 1994; Brechot *et al*., 2000). It is reasonable to expect that the temporal sequence of these genetic alterations in HCC may vary between regions with differing risk factors present. These hypotheses can now be tested at the individual level, by applying new technologies like detection of DNA mutations in cell-free plasma, to reveal the temporal sequence of the critical cellular and molecular events leading to HCC.

Materials and Methods

Study design and participants

A description of the design, recruitment procedures, and laboratory testing methods of the study has been previously reported (Kirk *et al*., 2004). Briefly, incident cases of HCC ($n=216$) and cirrhosis ($n=121$) were recruited from three tertiary hospitals sites in The Gambia from September 1997 to January 2001. HCC cases were defined based on pathological examination in 54 (25.0%) and by a combination of ultrasound lesions compatible with HCC and an α-fetoprotein (AFP) level >100 ng/ml in 162 (75.0%). Study participants diagnosed with cirrhosis met defined ultrasound criteria for cirrhosis (Lin *et al*., 1993) and had no focal lesions suggestive of HCC. Controls with no clinical evidence for liver disease ($n=408$) were frequency matched by age (within 10 years), gender, and study site. Subject evaluation included a clinical examination, an interview, and collection of blood specimens on all participants. Dietary intake of a variety of foods was assessed through a structured questionnaire administered by a trained interviewer in the local language. All study participants provided informed consent and both Gambian and international institutional review boards approved the study protocol.

Laboratory testing

Blood specimens were stored at -70°C until subsequent testing. AFP was quantified by standard radiometric assay methods (DiaSorin SA, Sallugia, Italy). HBV surface antigenemia (HBsAg) and HBV 'e' antigenemia (HBeAg) were determined as markers of chronic HBV carriage or viral replication, respectively, by standard laboratory kits (Murex Diagnostics Limited, Dartford, UK and Sorin Biomedica Diagnostics, Vercelli, Italy). HCV antibody status (anti-HCV) was tested by third generation ELISA (ORTHO, Neckargemund, Germany) with RIBA confirmation of reactive samples (CHIRON, Emeryville, CA, USA).

Detection of 249ser TP53 mutations

Circulating free DNA was extracted from 200 μl of plasma using QiAmp® DNA Blood Mini Kit according to the manufacturer's blood and body fluid spin protocol (Qiagen, Hilden, Germany). Purified DNA was eluted from the QiAmp Silica column with two volumes ($2\times 50\,\mu$l) of water (PCR-grade, Sigma, St Louis, MO, USA). DNA concentration was

measured using Picogreen dsDNA quantitation kit (Molecular Probes, Eugene, OR, USA). The fluorescence was read with a Fluoroskan Ascent FL fluorimeter (Labsystems, Helsinki, Finland). DNA (20 μl of resuspended, purified material) was used for amplification of exon 7 of the TP53 gene with 0.2 μM primers (final concentration) flanking this exon (sense: 5′-CTT GCCACAGGTCTCCCCAA-3′, antisense: 5′-AGGGGTCA GCGGCA AGCAGA-3′) using HotStarTaq polymerase (Qiagen). When necessary, a second PCR reaction was performed using nested primers (sense: 5′-AGGCGCACTGG CCTCATCTT-3′, antisense: 5′- TGTGCAGGGTGGCAAGT GGC-3′; Genset, Evry, France). Both PCR reactions involved a 15-min HotStarTaq polymerase activation at 95°C, from 40 to 60 cycles of denaturation (94°C, 30 s), primer annealing (60°C, 30 s), and extension (72°C, 30 s), followed by a final 5-min extension at 72°C. The size of the final PCR fragment was 177 bp.

PCR products (10 μl) were digested by *Hae*III restriction endonuclease (Boehringer Mannheim, Germany), which cuts within a GG|CC sequence encompassing codon 249 (AGG). Mutant, 158 bp fragments were visualized on 3% agarose gel stained with ethidium bromide, eluted, reamplified by PCR (5 μl of DNA, using same primers and conditions as above) and sequenced by automated, dideoxy sequencing (sequencer AbiPrism 3100, Perkin-Elmer). All results represent a minimum of two fully independent analyses. When discordant results were obtained in two successive analyses, a third analysis, including cutting and sequencing, has been performed and the consensus has been taken as the final result. At all steps of the procedure, a series of controls without DNA template were used to rule out contamination.

Statistical analysis

Dichotomous outcome variables (including 249ser TP53 mutation positive *vs* negative, cirrhosis *vs* control, and HCC case *vs* control) were sequentially analysed by similar methods. In univariate analysis, associations of outcome variables with independent variables were evaluated for statistical significance by Pearson's χ^2 and Fisher's exact tests. All *P*-values reported are two-tailed. In multivariable analysis, odds ratios (ORs) with 95% confidence intervals (CIs) were estimated as measures of association by stepwise unconditional logistic regression. With 249ser TP53 status as the outcome, study group was included in the modeling as an independent variable. Additional variables evaluated included: age, gender, recruitment site, recruitment season, place of residence, ethnicity, socioeconomic status variables, groundnut intake, HBV status, and anti-HCV status (data not shown). Data presented include variables found to be associated with 249ser TP53 status including study group, recruitment season, groundnut intake, HBeAg status, and alanine aminosparate (AST) levels. In multivariable analysis of HCC and cirrhosis risk, 249ser TP53 status was added to a previously developed unconditional logistic regression model (Kirk *et al*., 2004), which included age, gender, recruitment date, recruitment site, ethnicity, socioeconomic status variables (including educational level and living in an earthen floor dwelling), and HBV and HCV serologic status. To evaluate whether 249ser TP53 status was associated with biochemical, virologic, or ultrasonographic markers of disease stage, stratified analysis of HCC cases only was performed based on 249ser status. Analyses were performed using Stata statistical software (College Station, TX, USA).

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